Regulation of the blood-brain barrier in health and disease

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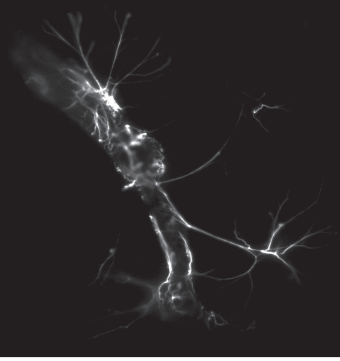
CHAPTER 1



General Introduction

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5. Thesis outline

1. The blood-brain barrier

The vasculature of the brain functions as a specialized barrier to protect the central nervous system (CNS) from the systemic circulation by restricting entry of unwanted molecules and immune cells into the brain, by active removal of cytotoxic compounds, and by supplying the brain with essential nutrients and oxygen through specific transport mechanisms. The blood-brain barrier (BBB) is not a rigid barrier but a dynamic structure that receives continuous input from the CNS cells it protects. This allows for a thorough response to the local demands for oxygen, nutrients, and buffering which is crucial for the maintenance of a CNS homeostasis that favours optimal neuronal function.

Several neuroinflammatory and neurodegenerative diseases like multiple sclerosis (MS), capillary cerebral amyloid angiopathy (capCAA), Alzheimer's disease (AD), epilepsy, and Parkinson's disease (PD) are associated with an impaired function of the BBB. Especially in MS, disruption of BBB function is paramount and an early marker for MS pathophysiology. The following introduction will cover various aspects of the BBB, including normal function, developmental aspects, functional disruption in MS, and finally, an introduction to the work described in this thesis.

1.1 Barrier properties of the brain endothelium

The BBB is composed of highly specialized brain endothelial cells (BECs) that line the CNS vascular wall. BECs form a tight structural barrier by the polarized expression of tight junction (TJ) proteins and a metabolic barrier by the expression of membrane efflux pumps. BECs are enclosed together with pericytes within the basement membrane onto which astrocytes firmly project their endfeet. Combined with the input of neurons and microglia this structure forms the neurovascular unit (NVU), which ensures optimal protection of the CNS from harmful compounds and the close regulation of CNS homeostasis. A schematic overview of the BBB in the healthy CNS is depicted in figure 1.

The BBB limits both transcellular and paracellular passage of cells and molecules from the systemic circulation into the CNS and vice versa. Transcellular passage of hydrophilic molecules is limited due to a low rate of transcytotic vesicles, an extremely low pinocytotic activity, expression of active efflux membrane pumps of the ATP-binding cassette (ABC) family such as P-glycoprotein, and high metabolic activity (cytosolic enzymes and transporters). To buffer excess amounts of neurotransmitters like glutamate from the CNS, BECs possess excitatory amino acid transporters (EAAT) 1-3, to limit neurotoxicity. In order to closely regulate the influx of only those components that are necessary in the CNS, BECs harbour specific transporters that actively transport nutrients like glucose into the CNS by glucose transporters (Glut1-3).

Paracellular diffusion of hydrophilic molecules and trafficking of immune cells is restricted by a network of TJ complexes which allow firm adhesion of BECs to each other and sealing of the inter-endothelial space[1-4]. Adjacent BECs express continuous rows of transmembrane proteins that make homophilic contact in the intercellular space and form TJs[5]. Claudins and occludin are the most important membranous components of TJs, but the participation of junctional adhesion molecules (JAMs) and adherens junctions (Cadherins) are important as well[1]. The C-terminal cytoplasmic domain of occludin, the first described TJ protein, is associated with the hence named zona occludens (ZO)-1 and ZO-2 proteins, which link occludins to the cytoskeleton. Claudins make up a family of proteins that consist of at least 23 closely

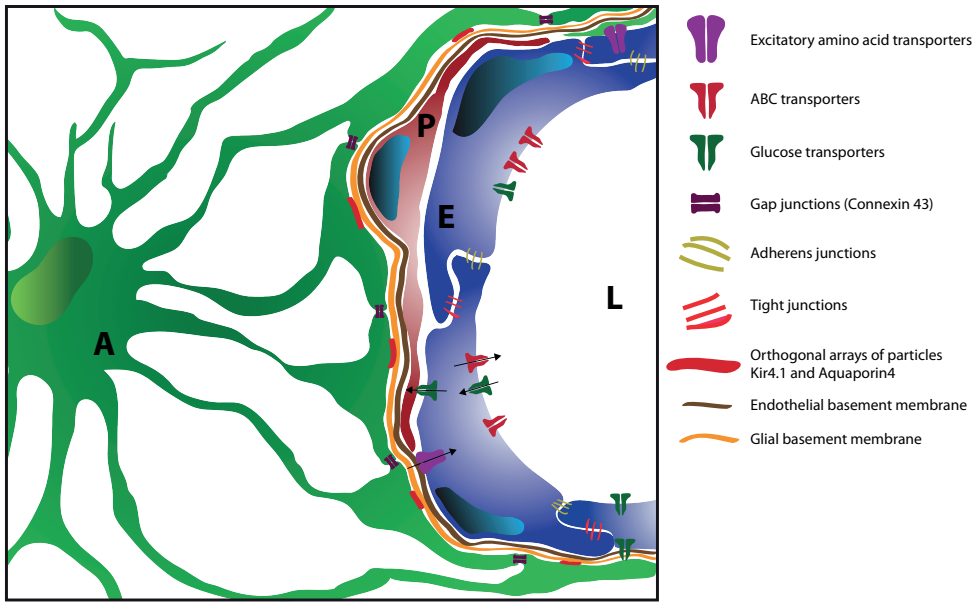

Figure 1. Schematic overview of the blood-brain barrier in the healthy CNS.
The endothelial cells (E) of the BBB are interconnected via tight and adherens junctional complexes and express various uptake (GLUT) and efflux (ABC-transporters, EEAT) transporters to regulate the flow of nutrients, metabolites, and harmful serum-components. Pericytes (P) tightly interact with the endothelial cells and both are encapsulated by the endothelial basement membrane. Astrocytes (A) project their processes to the endothelial basement membrane that form endfeet structures. These endfeet form the glial basement membrane and completely envelop the CNS microvasculature. Astrocyte endfeet form orthogonal arrays of particles at the glial basement membrane which incorporate high levels of ion and water channels, thereby regulating local osmotic homeostasis. Astrocyte endfeet form gap junctions with neighbouring endfeet which allows for rapid communication and exchange of metabolites. The local release of trophic factors further ensures normal brain endothelial cell function.

related members. At the BBB, the presence of claudin-1,-3-, 5 and recently -12 has been reported[6, 7]. The endothelium of the CNS microvasculature shows a high degree of specialization to form the BBB, and the regulatory process behind this specialization is still largely unknown. However, in the past decade, a number of pathways involved in the development and maintenance of the BECs have been put forward.

1.2 Astrocytes

Astrocytes are strongly represented within the neurovascular unit, ensheathing over 95% of the abluminal microvascular surface. It was this observation that gave rise to the idea that astrocytic processes formed the BBB, until electron microscopic studies showed that BECs were responsible for barrier function in brain microvasculature[8].
Astrocytes are able to influence a number of features of the BECs, leading to increased integrity of the BBB. TJ expression and TJ complex formation and maturation, expression and localization of BEC transporters, and specialized enzyme systems have been shown to be upregulated under astrocyte influence[9]. The notion that astrocytes can induce and maintain BBB properties in BECs through physical interaction and secreted agents has been

widely accepted[10]. Astrocyte processes extending towards CNS microvessels terminate in specialized (perivascular) endfeet structures onto the basal lamina surrounding the BECs. Astrocyte endfeet associated with BECs show a high density of orthogonal arrays of particles (OAPs), organized arrays of ion- and volume-regulating membrane particles identified by freeze fracture[11], containing channels like the water channel aquaporin-4 (AQP4) and the potassium ion channel Kir 4.1[12]. Membrane proteins in OAPs represent a strong polarization of perivascular astrocyte function and correlate with the expression of the basement membrane molecule agrin, an important proteoglycan for BBB integrity[13], responsible for the correct localization of AQP4. The distribution of these channels in OAPs is most likely important in the regulation of BBB homeostasis, as disruption of this distribution is associated with microvascular damage in, among other pathologies, AD[14].
The observation of astrocyte conditioned medium inducing junction formation in BECs *in vitro*[15] gave rise to the idea that astrocyte-derived secreted factors were able to influence the BBB properties of BECs. Numerous astrocyte-derived agents have since then been described, mainly by *in vitro* studies, as modulators of BEC barrier function. Transforming growth factor-β (TGFβ) secreted by astrocytes has been shown to mediate the regulation of tissue plasminogen activator and the anticoagulant thrombomodulin[16]. Glial-derived neurotrophic factor (GDNF) has been found to enhance barrier function in BECs through the induction of TJ expression[17]. Fibroblast growth factor (FGF) was found to decrease BBB permeability[18], consistent with the observation that FGF knockout mice show decreased levels of TJ proteins and BBB integrity loss[19] and Angiopoietin-1 (ANG1) was shown to be an astrocyte secreted factor that increases TJ expression of BECs with an important effect on BBB permeability[20]. Recently, sonic hedgehog (Shh), a member of the Hh pathway, was shown to be produced and secreted by perivascular astrocytes in the human and mouse adult brain and that microvascular BECs expressed the receptors and the intracellular machinery to respond to Hh ligands[21]. Pharmacological neutralization of Hh receptors or genetic deletion of Hh receptors lead to enhanced permeability of the BBB and loosening of the TJs. Together, these observations confirm the important role of perivascular astrocytes in the regulation of the BBB in the adult CNS.

1.3 Other cell types involved in BBB function

Although astrocytes are the most abundant cell type associated with the endothelial cells of the BBB, interactions between the BBB endothelium and other CNS cell types have been described to work in concert with astrocytes and endothelial cells in the regulation of the NVU.

Neurons

Due to the high metabolic need of neurons and the dynamic pattern of neural activity, the CNS requires a tight regulation of the microcirculation which provides the necessary nutrients and means of waste transport. The coupling of brain activity and CNS blood flow is therefore crucial for normal neuronal functioning. Although the cellular aspect of this coupling is not fully understood, the involvement of all components of the neurovascular unit seems to be necessary for the regulation of CNS blood flow by neurons[22]. Besides the indirect regulation of blood flow, neurons are also found to directly innervate BEC or BEC-associated astrocytes functioning as a liason for neuronal-endothelial coupling. Because disruption of BBB integrity

is often found to accompany pathological changes in CNS blood flow, it was suggested that the observed BBB permeability changes were due to active involvement of neurons in BBB integrity[23]. Indeed, noradrenergic[24], serotonergic[25], cholinergic[26], and GABA-ergic[27] neurons have been found to directly contact the microvascular endothelium. Although the mechanism of action is unknown, neurons innervating the neurovascular unit are thought to regulate BBB permeability[28, 29]. An example of this regulation is shown by the loss of cholinergic innervation of the CNS microvasculature, resulting in impaired cerebrovascular functioning in AD[26]. In short, neurons in the NVU do not only play an active part in the regulation of CNS blood flow, but also seem able to directly influence BBB permeability, through direct innervations of BECs.

Pericytes

Pericytes are perivascular, contractile cells that closely associate with capillary walls, and directly contact the BEC membrane[30]. Pericytes are thought to exert influences on the BEC, through their specialized junctions, involving gap junctions, TJs, and AJs[31, 32]. Although the molecular mechanism by which pericytes mediate vascular integrity is not yet understood, perivascular pericytes are known to release growth factors and angiogenic molecules which are able to regulate microvascular permeability and angiogenesis[33]. Besides influencing BEC function, pericytes also contribute to the stability of microvessels and cover a large part of the abluminal BEC surface, further influencing BBB permeability[31, 34]. The regulation of blood flow in CNS capillaries by pericytes has been shown to result from pericytes contracting and relaxing in a regulated manner[35].

Reductions in the number of CNS pericytes have been linked to neurovascular disruption in both AD[36] and ALS[37] but the mechanism of pericyte detachment or disappearance from the BBB remains unknown. Furthermore, pericytes play a large role during BBB development, which will be discussed in the following chapter.

2. Development of the blood-brain barrier

The vascular system of the CNS arises early in embryogenesis through the invasion of vascular plexus-forming angioblasts into the head region[38], followed by invasion of the CNS by vascular sprouts from the peri-neural vascular plexus, extending towards the ventricles[39]. Peripheral vascular system development has been described in detail and various signalling systems taking part in vasculogenesis, angiogenesis, and differentiation have been uncovered[40-42]. However, few reports exist on developmental CNS-specific cues for the induction of the specialized EC phenotype found at the BBB. CNS cells surrounding the endothelial layer are thought to provide angiogenic ECs with the appropriate signals for BBB maturation[43], as well as with the signals required for maintenance of the mature BBB-phenotype. The different cell types involved in BBB development during embryogenesis are described below.

2.1 Astrocytes and radial glia

During CNS development, radial glial cells provide structural and trophic cues[44] and in the later stages of development differentiated astrocyte endfeet projections provide an almost complete enveloping of the brain microvasculature in adult vertebrates[45]. The search for CNS-specific signals which affect the BBB phenotype in brain ECs has implicated astrocytes and glial progenitors as inducers of a specific BBB-phenotype in brain EC[46]. Interestingly, the

developmental window in which the vasculature invades the developing CNS and matures into the BBB overlaps with the induction of neuronal differentiation and outgrowth[47] and both systems share guidance cues and differentiation-inducing signaling pathways[48]. Radial glial cells have a prominent role in neuronal differentiation and outgrowth, leading to the hypothesis that these cells also function as BBB-inducing cells during development. This is illustrated by the fact that until recently, sonic hedgehog (Shh)-signaling in the CNS has been mostly associated with neuronal patterning and differentiation[49, 50]. However, Shh was shown to be released by fetal astrocytes, and able to induce BBB-properties in ECs during embryonic CNS development[21]. This recent finding paves the way for the investigation of other well-known neuronal differentiation signals, in view of BBB development. Retinoic acid (RA) is a powerful Vitamin A-derived morphogen in early CNS development and radial glia-derived RA is crucial in normal neurogenesis[51]. The involvement of RA in BBB development during CNS embryogenesis is described in this thesis.

2.2 Pericytes

Pericytes have recently emerged as a major contributor to the development of the BBB in murine studies, showing increased permeability of the BBB and dysregulation of junctional protein localization in the microvasculature of pericyte-deficient mice[52, 53]. Although contradictory evidence exists regarding the induction of BEC-specific gene expression patterns by pericytes, the presence of these perivascular cells, thought to originate from neural crest cells, is crucial in the very early stage of BBB development. Since many developmental events occur in parallel at the immature BBB, it seems likely that the interplay of radial glia, pericytes, and neural progenitors is needed for the correct patterning and subsequent maturation of the BBB. Interestingly, pericyte-deficiency during development also leads to mislocalization of astrocyte-endfeet on the endothelial basal lamina and a disturbed polarization of endfeet-specific proteins[53].

2.3 Neural progenitors

CNS-specific Wnt/β-catenin signaling has been firmly implicated in normal BBB-development. Animal models in which β-catenin activation was ablated showed decreased BBB-maturation and increased permeability, whereas no effects were reported on the non-CNS vasculature[54-56]. Furthermore, the expression of Wnt-ligands associated with canonical β-catenin activation in ECs where shown to be region dependant and only detectable in neural progenitors. Another function that has recently been attributed to neural progenitors is the regulatory role in Notch signaling during CNS development[57], which in turn has been implicated in the stabilization of pericyte-endothelial interactions in the CNS[58].

A better understanding of all developmental systems converging on BECs during BBB development does not merely answer fundamental scientific questions. New insights in BBB development might also provide new avenues of research in neurological disorders with a known involvement of BBB disruption. Regeneration of the barrier with existing developmental pathways could prove to be an interesting field for future therapeutic strategies.

3. Multiple Sclerosis

Disruption of BBB function has been implicated in numerous CNS diseases, and might underlie several pathophysiological processes leading to CNS disorders. In MS pathophysiology, BBB disruption is an early clinical indicator of neuroinflammation, and eventually, MS lesion formation. This clear indication of BBB damage with MS pathology has lead researchers to investigate the molecular mechanisms behind BBB disruption, the extent of damage caused by an impaired BBB, and finally, possible strategies to repair the damaged BBB in order to limit neuroinflammation.

3.1 Clinical features and diagnosis

Multiple sclerosis (MS) is a chronic inflammatory disorder of the CNS. MS is characterized by the presence of focal inflammatory lesions scattered throughout the brain. Depending on temporal stage, lesions are hallmarked by inflammation, demyelination, gliosis, axonal injury and diffuse axonal degeneration[59, 60]. The global median estimated prevalence is 30 per 100.000, resulting in over two million people affected with MS worldwide. With an average age of onset between 25 and 32 years of age, MS is one of the most common neurological disorders and causes of disability in young adults[61].

Presentation and symptoms of MS are characterized by great variability and diversity. In general, the initial symptoms and signs are sensory impairment, optic neuritis, motor deficits, limb ataxia and difficulty with balance[62]. The majority of MS patients are subject to a relapse with onset of MS, referred to as clinically isolated syndrome (CIS), which may eventually convert to MS[63]. The clinical manifestation of MS varies and can be described by three clinical course definitions: Relapsing–remitting (RR) MS, accounting for the onset of disease in about 85% of MS patients, is described by clearly defined disease relapses with full or partial recovery. Secondary-progressive (SP) MS is described by initial RR disease course, followed by progression with or without occasional relapses, minor remissions, and plateaus[64]. Primary–progressive (PP) MS, accounting for the onset of disease in about 10% of MS patients, is described by rapid disease progression from onset with occasional plateaus and temporary minor remissions.

Diagnosis of MS is primarily based on clinical grounds, comprising neurological exams and clinical history. If a diagnosis based on clinical presentation is not possible, radiological and laboratory assessments such as magnetic resonance imaging (MRI) and cerebrospinal fluid (CSF) analysis may be essential for diagnosing MS. MRI analysis detects MS lesions in brain and in spinal cord and can therefore provide evidence of dissemination of MS lesions in both time and space, two potential criteria for diagnosis of MS. CSF analysis may provide supportive evidence by the presence of CNS-derived antibodies (oligoclonal bands).

3.2 Aetiology

The precise aetiology of MS remains unknown. Epidemiological studies indicate that environmental factors may contribute to the development of MS[65], but that development of MS will probably arise in the genetically susceptible population, upon exposure to environmental factors[66].

Family studies have revealed that first degree relatives of MS patients are more likely to develop MS compared to non-related individuals[65, 67]. Further support for a genetic risk factor for MS susceptibility derives from twin studies, which show a higher concordance rate

of MS in monozygotic twins compared to dizygotic twins[68-70]. Genetic associations with MS include certain human leukocyte antigen (HLA) alleles, genetic mutations or polymorphisms in genes coding for cytokines, cytokine receptors, adhesion molecule, and co-stimulatory molecules[71].
Environmental risk factors for MS are diverse of character. Several infectious pathogens such as varicella zoster virus, herpes viruses, chlamydia, and the Epstein-Barr virus are described as environmental risk factors. Two important risk factors amongst the non-infectious environmental risk factors for MS are latitude and vitamin D. Populations living at higher latitude show an increased prevalence of MS compared to populations living near the equator. A finding most likely associated with vitamin D serum levels. Interestingly, studies show that populations living at high latitude but with rich vitamin D food intake also show reduced MS prevalence[66, 72]. Pinpointing MS aetiology has thus far proved elusive. Therefore, understanding the mechanisms of disease in MS might result in an enhancement of the current therapeutic strategies to combat the progression of MS.

3.3 Pathogenesis

A distinct feature of MS pathology is the formation of demyelinated lesions, or plaques, in the CNS. According to De Groot /van der Valk staging, MS lesions can be classified as pre-active, active demyelinating, active but not demyelinating, chronic active, and chronic inactive lesions[73]. Pre-active lesions may be located near existing demyelinated plaques and in normal appearing white matter. The lesions do not show demyelination but are characterized by modest white matter abnormalities including clusters of activated microglial cells and few perivascular leukocytes. In addition to the minimal leukocyte infiltration, there is a relative absence of demyelination and discrete basement membrane abnormalities. In contrast to preactive lesions, active demyelinating lesions are characterized by loss of myelin and presence of abundant macrophages containing myelin degradation products. In addition, parenchymal and perivascular infiltrates of macrophages and lymphocytes are observed as well as abundantly present reactive astrocytes. A chronic active MS lesion is a demyelinated lesion containing a hypocellular center and a hypercellular rim of hypertrophic astrocytes, microglia, and macrophages[74]. Finally, chronic inactive lesions are demyelinated and hypocellular with only moderate expression of major histocompatability complex class II (MHCII) and few lipid-phagocytosing macrophages present[75].
Complementary to demyelination, axonal damage is known to be of great importance in MS pathology. Early axonal damage is found at areas of acute demyelination and inflammation [76, 77]. Axonal loss has been shown to be a major cause of irreversible neurological disability in MS[78]. The irreversible nature of axonal damage and its association with inflammation suggest that anti-inflammatory treatment should be utilized early and that future therapies could benefit from the inclusion of a neuroprotective component to prevent neurological deterioration.
Despite many advances in both molecular and clinical MS research, MS still remains incurable. Nevertheless, various therapies for treatment of MS are available and more therapies will most likely become available in the following years. Current MS therapies are limited to reduction of relapse rates, slowing down disease progression, accelerating recovery of relapses, and palliative treatment.

4. The BBB in MS

In MS pathology, numerous changes in BBB structure and functioning have been described. These observations derived from *in vitro* systems, animal models, and patient tissue studies, show a high involvement of the disruption of BBB integrity and function in MS pathophysiology. The combined outcome of these studies has led to the notion that BBB disruption represents an early event in MS lesion formation, preceding both the massive infiltration of leukocytes (mainly T lymphocytes and monocyte-derived macrophages) and nervous tissue destruction[79]. Even before clinical symptoms arise, MRI scans of animals with experimental allergic encephalomyelitis (EAE), a well-established and validated animal model for the inflammatory phase of MS, show leakage of the BBB before leukocyte infiltration[80]. However, before leukocytes adhere and transmigrate through the BBB, the cerebral endothelium must be activated by inflammatory mediators which induce expression of cell adhesion molecules (CAM) on BEC, with which leukocytes interact. A better understanding of the molecular changes occurring at the BBB during MS pathophysiology could shed light on the crucial steps needed for the breach of the barrier in MS, eventually leading to a better understanding of the mechanism that can be utilized to halt the inflammatory component of MS. A schematic overview of the inflammatory changes at the BBB described below is depicted in figure 2.

4.1 Inflammation at the BBB in MS

Tumour necrosis factor α (TNF-α) and chemokine (C-C motif) ligand 2 (CCL2) are two examples of numerous pro-inflammatory molecules which cause an upregulation of endothelial CAMs such as E-selectin, P-selectin, vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1)[81], activated leukocyte cell adhesion molecule (ALCAM)[82] and melanoma cell adhesion molecule (MCAM)[83]. While it remains unclear what triggers initial vascular activation in MS, reactive astrocytes and perivascular microglia are potent contributors to endothelial inflammation since they secrete pro-inflammatory cytokines and chemokines such as TNF-α, interleukin (IL)1β, IL6, IL12, and CCL2 during the disease process[84-86]. Through secretion of pro-inflammatory molecules, astrocytes and microglia not only contribute to direct disruption of the BBB, but also facilitate upregulation of CAMs and form a chemo-attractive gradient, thereby promoting recruitment and adhesion of more leukocytes to BECs.

Inflammation driven tissue damage in the CNS of MS patients is driven by both autoreactive, antigenic CD4 T cells and CD8 T cells[87-94]. In addition, IL17 producing memory CD4 T cells (Th17 cells) are found within active MS lesions[95]. Of the antigen presenting cells (APCs), infiltrated monocyte-derived macrophages are thought to possess a crucial role in orchestrating processes such as demyelination and axonal damage[96-100].

Before entering the CNS, leukocytes have to transmigrate through the specialized ECs of the BBB. Monocytes, the effector cells within MS lesions, are attracted to the perivascular space in high numbers. Within the process of monocyte trafficking across the BBB, it has been demonstrated that reactive oxygen species (ROS) play a dominant role. ROS are produced by monocytes upon firm adhesion to ECs and subsequently enhance migration and adhesion of monocytes[101]. Treatment of EAE animals with antioxidants such as flavonoids and lipoic acid suppressed the development of EAE by lowering the entry of inflammatory cells into the CNS. Histological examination demonstrated a reduced number of infiltrated T-cells and

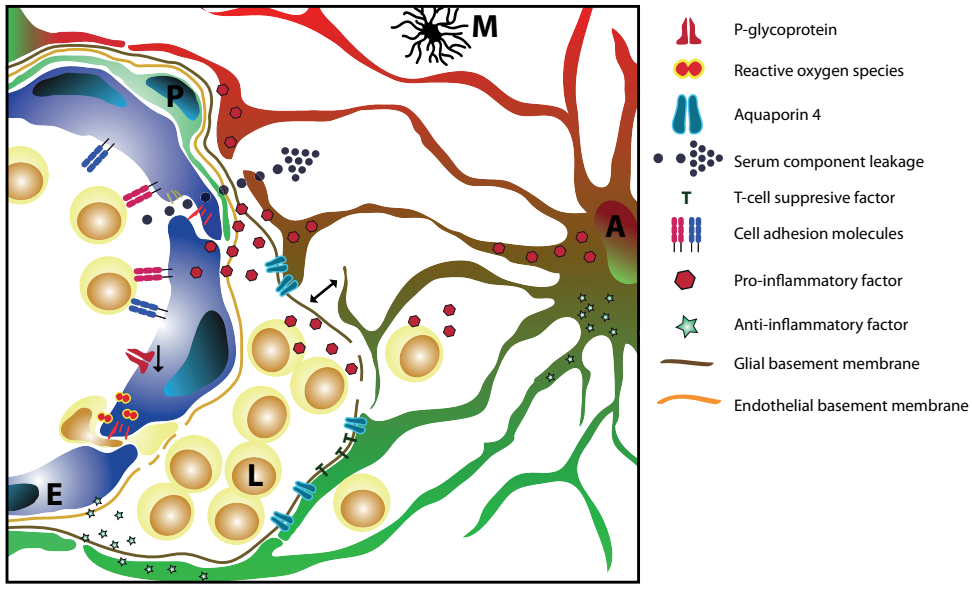

Figure 2. Neuroinflammatory changes at the blood-brain barrier.
During neuroinflammation in MS, the inflamed BBB shows loss of barrier integrity, resulting in leakage of serum components into the CNS. Endothelial cells (**E**) express cell adhesion molecules leading to adhesion and migration of activated leukocytes (**L**) incto the CNS. Leukocytes locally release reactive oxygen species to disrupt TJ-complexes. Efflux transporter P-glycoprotein expression is decreased on endothelial cells. Reactive astrocytes (**A**) and activated microglia (**M**) contribute to the neuroinflammatory process by releasing pro-inflammatory chemokines and cytokines. Aberrant astrocyte endfeet aquaporin 4 expression is thought to aggravate BBB-dsiruption. The protective role of reactive astrocytes is illustrated by expression of T-cell suppressive factors and the release of anti-inflammatory factors like sonic hedgehog. The role of pericytes (**P**) in inflammatory BBB-disruption is not known, although the loss of pericytes is associated with the damaged BBB.

macrophages, suggesting a role for ROS in BBB permeability[102, 103]. Moreover, it was shown that super oxide is the predominant ROS type that induces BBB disruption by inducing TJ rearrangements and cytoskeletal changes, allowing cell migration[104].

4.2 Immune cell trafficking across the brain endothelium

The transmigration of leukocytes across the vascular wall requires the sequential activation and interaction of numerous molecular effectors expressed by BECs and immune cells, including selectins, chemokines, adhesion molecules of the immunoglobulin superfamily and their integrin counter ligands. The importance of leukocyte migration in MS is highlighted by the fact that the healthy CNS is devoid of immune cells and has been further demonstrated by the clinical efficacy of pharmacological blockers of migration in human MS patients. Interfering with leukocyte extravasation and diapedesis by blocking the adhesion cascade has indeed proven to be beneficial in reducing clinical disease activity and pathological indices in MS. Natalizumab, which blocks VLA-4, the ligand of VCAM-1, is reported to reduce migration of most leukocyte subtypes into the brain. Therefore, validation of the biological importance and of the clinical relevance of immune cell trafficking in MS is provided by the important clinical benefit of anti-VLA-4 blocking therapies. These VLA-4 blocking strategies

prevent immune cell recruitment to the CNS, reduces myelin and axonal damage and alleviates clinical symptoms and disease progression in both animal models of MS[105] and in MS patients[106].

4.3 Astrocyte-endothelial interactions in MS

During MS pathogenesis, reactive astrocytes participate in various mechanisms that contribute to neuroinflammation. Reactive astrocytes aggravate inflammation by increasing vascular activation and leukocyte accumulation in the CNS, and are involved in loss of BBB integrity, possibly mediated by local release of pro-inflammatory molecules like IL-1β, IL6, and CCL2[107-109]. Furthermore, the involvement of the sphingomyelin metabolism in the form of pro-inflammatory ceramide production by reactive astrocytes in MS lesions has been reported. Ceramide was found to impair the function of the BBB in vitro [110], illustrating the impact of the reactive astrocyte phenotype on the barrier properties in MS. In the same study reactive astrocytes were found to have an induced expression of sphingosine-1-phosphate receptors which, after triggering with the S1P analogue Fingolimod (FTY-720P), a drug currently used in the clinic for MS treatment, resulted in a diminished production of pro-inflammatory mediators[110, 111]. Together, these data indicate that the dampening of the reactive astrocyte phenotype is an attractive new therapeutic strategy[112].

In addition, once inflammation has abated, astrocytes are the major cell type involved in glial scar formation and are thereby directly associated with inhibition of axonal regeneration[113]. In contrast, during pathophysiology, astrocytes may also exert protective properties and promote cellular regeneration. Astrocytes are able to produce antioxidant enzymes and glutamate metabolizing enzymes and transporters suggesting an important role in scavenging reactive oxygen species (ROS) and extracellular glutamate[114, 115]. Furthermore, reactive astrocytes maintain the capacity to secrete T-cell suppressive factors[116], anti-inflammatory cytokines, and neurotrophic factors[117]. Finally, astrocytes in active MS lesions produce semaphorins, which are known to form chemotactic gradients for developing oligodendroglial cells, thereby possibly promoting remyelinisation[118]. This accentuates the important and dual role of astrocytes in CNS damage, which is not limited to BBB damage, but encompasses all neuroinflammatory changes in the CNS. Inflammatory changes affecting the interaction between astrocytes and the BBB in MS are described below.

The Hedgehog pathway

Neuroinflammatory conditions such as MS are associated with a breakdown of the BBB. A recent study showed that human astrocytes treated with TNFα and IFN-γ increased Shh expression and that BECs grown in astrocyte-conditioned media (ACM) and treated with TNF and IFN-γ increased their expression of Hh receptors Ptch-1 and Smo[21]. Addition of Shh to BEC cultures induced a reduction in both CAM expression and chemokine secretion. Within control brain tissue and normal-appearing white matter (NAWM) obtained from MS brains, astrocyte processes and endfeet surrounding parenchymal vessels displayed Shh immunoreactivity. However, Shh immunoreactivity was strikingly enhanced in hypertrophic astrocytes and processes throughout active demyelinating MS lesions, and the Hh transcription factor Gli-1 was increased in BBB-ECs[21, 119]. Upon inflammatory stimulation, astrocyte-secreted Shh therefore induces expression of Hh receptors in BECs, which leads to the translocation of the Hh transcription factor Gli-1 into the nucleus of BECs. The hedgehog

pathway, where Hh ligands are secreted by astrocytes and Hh receptors are expressed by BECs, therefore acts as a molecular repressor of CNS inflammation and promotes BBB repair.

Aquaporin-4 and Kir4.1 in astrocyte endfeet

Astrocytes with endfeet terminating in the neurovascular unit perform specific functions in the maintenance of perivascular ion and water homeostasis[120]. Extracellular potassium ions released by neurons require spatial buffering by astrocytes to maintain homeostasis. The inwardly rectifying Kir4.1 potassium channels which are highly expressed in the polarized astrocyte endfeet meet this need for potassium buffering. Potassium ion buffering by astrocytes is accompanied by osmotic changes and slight cell swelling. The AQP4 water channels present at high densities in the OAPs of astrocytic endfeet regulate these osmotic changes by redistribution of excess water. The tight regulation of expression and distribution of the ion and water channels on astrocytic endfeet is necessary for homeostasis and disruption of this compensatory system has been shown for BBB disruption in Alzheimer's disease[14] and glioblastomas[121], both involving aberrant agrin expression. The increase of AQP4 expression observed in brain edema, probably serving as an adaptive mechanism, tends to aggravate the BBB disruption[122]. AQP4 upregulation has also been shown in reactive (hypertrophic) astrocytes in response to injury, correlating with BBB disruption[123]. Reactive astrocytes in MS lesions were shown to have increased levels of AQP4 expression[124], which could possibly contribute to further edema-induced BBB damage after initial disruption.
The observation that the astrocytes with the highest AQP4 expression are located at the outer rim of active MS lesions, resembling ischemic foci[125], suggests that altered AQP4 expression, localization, or regulation by agrin could be contributing to aggravation of MS pathology.

Connexin 43

Astrocytes in the neurovascular unit are coupled together via gap junctions (GJ), mainly formed by connexin43 (Cx43)[126]. The coupling through GJ provides the network of astrocytes with a cytoplasmic continuity which allows the free and fast passage of (signaling) ions and metabolites between astrocytes. This syncytium of cells provides the BBB with a network of continuously communicating astrocytes, where fast responsiveness can be crucial in maintaining homeostasis.
In EAE a decrease in astrocytic Cx43 expression was observed in the inflammatory regions of EAE pathology, suggesting a decreased astrocytic connectivity in these areas[127]. Whether reduced astrocyte-astrocyte communication during inflammation is detrimental or beneficial remains to be determined, although the possible involvement of Cx43 in maintaining BBB integrity through co-localization with TJ-proteins in porcine BEC has recently been reported[128]. The effects of the loss of GJ-contact between astrocytes on astrocyte activation, BBB integrity, and inflammatory response should be investigated further to address the impact on MS pathology.

P-glycoprotein

The drug-efflux transporter P-gp is an ATP-dependent efflux pump highly expressed on the luminal side of BEC, responsible for the active removal of a broad range of hydrophobic molecules from the BEC cytoplasm[129]. P-gp function leads to the prevention of potentially

neurotoxic molecules entering the CNS tissue, also leading to the low penetration of CNS-therapeutical drugs[130]. The expression of P-gp is not confined to BECs, but expression was also shown to localize in astrocytic endfeet structures[131]. In a recent study, P-gp expression in the inferior colliculus was shown to be heavily reduced in BECs, following a chemically induced focal loss of astrocyte contact. Interestingly, P-gp expression returned to normal when astrocytes were seen to repopulate the affected area[132]. This observation indicates a role for astrocytes in the induction and maintenance of P-gp expression by BECs.
In MS lesions, a significant reduction of microvascular P-gp expression compared to NAWM has been reported, suggesting that a loss of P-gp expression might be involved in lesion formation or aggravation[133].
Altogether, astrocytes show a high degree of control of BBB function, both under healthy and disease conditions. Despite the fact that altered astrocyte-endothelial interaction might contribute significantly to MS pathogenesis, this role is far from understood. A better understanding of the changes that are related to astrocyte-endothelial crosstalk will enhance our ability to intervene in their communication in future therapeutic approaches.

5. Thesis aims and outline

The involvement of BBB disruption in MS pathophysiology has been extensively investigated and is well documented. Not only do we know that BBB functions such as low trans- and paracellular permeability and high drug resistance are decreased in MS lesions, but the immune activation of BECs by a pro-inflammatory environment has also been investigated thoroughly. Our understanding of BBB damage and activation in MS has greatly expanded in the recent decade. Although it is now widely recognized that blocking the interaction of leukocytes with the BBB in MS is a highly efficient therapeutic strategy, it has not yet led to a great expansion of research aimed towards protective or repair strategies that target the damaged BBB. **Therefore, the aim of this thesis is to document the changes that occur at the damaged BBB in MS, with a focus on altered endothelial-astrocyte interactions that might serve as potential therapeutic targets.** The research described in this thesis will contributes to a better understanding of the BBB changes in MS and provides a new BBB-developmental mechanism with possible therapeutic potential. In **chapter 2** we describe a novel role for fetal astrocyte-derived retinoic acid (RA) in the induction of the BBB during CNS development, further expanding the list of BBB-inducing signals. **Chapter 3** describes our findings on BBB alterations in the animal model for MS, EAE. We show that BECs in inflammatory brain lesions re-express the non-CNS endothelial cell marker plasmalemmal vesicle associated protein (PLVAP), which is not expressed on BECs under normal conditions. This suggests a dedifferentiation of immune-activated BECs. **Chapter 4** covers our advances in the investigation of altered endothelial-astrocyte interactions. We show that reactive astrocytes in MS lesions show enhanced expression of ABC-transporters, which leads to enhanced immune cell migration *in vitro.* In **chapter 5** we show that reactive astrocytes in MS lesions respond to inflammation by re-expressing the enzyme retinaldehyde dehydrogenase (RALDH), leading to the production of RA. Furthermore, we describe the anti-inflammatory and protective effects of RA on inflamed BECs. Finally, all the chapters listed above will be discussed, as well as their implications and future perspectives, in **chapter 6**.

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